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Antifungal and phytochemical screening of some Nigerian medicinal plant extracts against toxigenic *Aspergillus flavus*

Y.A. Jeff-Agboola^{1*} and L.B. Awe²

Abstract: In vitro study of ethanolic, cold and hot-water extracts of Cymbopogon citratus, Moringa oleifera, Ocimum gratissimum and Clerodendrum volubile was determined against toxigenic Aspergillus flavus. The antifungal efficacies of the medicinal plants were investigated using agar well diffusion technique and minimum inhibitory concentration. The phytochemical constituents of the plants were also investigated. It was observed that, hot-water extract of C. citratus was most effective against toxigenic A. flavus, cold water extract of M. oleifera was effective against toxigenic A. flavus, the most antifungal effect of O. gratissimum and C. volubile were efficient using ethanolic extraction method. One thousand milliarams per millilitres concentration of these plant extracts had inhibitory effect on toxigenic A. flavus. The results of phytochemical screening of the plant extracts for the detection of volatile oil, glycoside, tannins and saponins shows that saponin and tannin, were detected in the ethanolic, cold and hotwater extract of M. oleifera, O. gratissimum and C. volubile. These result of the research indicates that ethanolic extracts of M. oleifera, O. gratissimum and C. volubile are used to cure infections caused by toxigenic A. flavus and the extracts may have role as pharmaceutical and preservatives.



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ABOUT THE AUTHOR

Y.A. Jeff-Agboola holds a PhD in Food Microbiology. Her main fields of research are food microbiology, food/feed mycotoxicology and food safety/ security. The reseach reported in this journal is part of her food safety and security research work as one of the measures of controling aflatoxin in food. She is working with smallholder farmers and always organising programs in the society with the aim of using innovative programs to benefit the rural poor community for wealth creation and contributing to improving food security status of the country through her applied research. She is currently a lecturer in the Department of Biological Sciences, University of Medical Sciences, Ondo Nigeria. Throughout her career she has had a number of stays in research centres. She has contributed to many congresses, panel discussions and proceedings, and is a widely published author.

PUBLIC INTEREST STATEMENT

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Wide varieties of plants are in use by conventional, medicinal and traditional medicine in south-west Nigeria to improve and sustain health and to treat minor illnesses. In the world, plants traditionally are used in oral health and to treat many diseases especially infectious diseases. With increasing number of fungal resistant to various antifungal, several attempts to use the antifungal potential of plants have been carried out. This perspective article describes the efficacy of some bio-active components of selected medicinal plants against toxigenic Aspergillus flavus known for the production of aflatoxin. Therefore, the study was designed to analyse and determine the effectiveness of four medicinal plants (Cymbopogon citratus, Moringa oleifera, Clerodendrum volubile, Ocimum gratissimum) against toxigenic Aspergillus flavus, evaluate the minimum inhibitory concentration of the plant extracts against toxigenic Aspergillus *flavus* and investigate the phytochemical properties of the plant extracts for clinical evaluation, pharmaceutical, cosmetic, agriculture and food industry utilisation.





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Subjects: Agriculture & Environmental Sciences; Bioscience; Botany; Environment & Agriculture; Food Additives & Ingredients; Food Science & Technology; Microbiology; Mycology; Pharmaceutical Science; Toxicology

Keywords: medicinal plant extracts; toxigenic Aspergillus flavus; antifungal; phytochemical

1. Introduction

Wide varieties of plants are in use by conventional, medicinal and traditional medicine in South-West Nigeria to improve and sustain health and to treat minor illnesses (Anderson et al., 2000). These plants are also used as flavours and spices in several refreshing foods, drinks and daily meals. More specifically, some of these plants are often used to lower cholesterol as anti-inflammatory, antioxidant, antifungal and neuroprotective against cerebral insufficiency in the elderly or as chemo-preventive agents; some of these products are also consumed to boost the immune system. Some are used as aphrodisiac; some give heartburn relief, migraine relief, and weight loss (Newman, Yang, Pawlus, & Block, 2008).

Plants are still a potential source of medicinal compounds. In the world plants traditionally are used in oral health and to treat many diseases especially infectious diseases including diarrhoea, fever and cold (Odenholt, Lowdin, & Cars, 2001), in addition, many recreational compounds used in traditional medicine have plant root.

According to World Health Organization (WHO) definition, a medicinal plant is a plant that can be used for therapeutic purposes and or its compound can be used as a pioneer in the synthesis of semi-synthetic chemical drugs (World Health organization [WHO], 1979).

With increasing number of fungal resistant to various antifungal, several attempts to use the antifungal potential of plants have been carried out. Antifungal compounds obtained from plants with different mechanisms of action against resistant microbial strains are of clinical importance (Eloff, 1999; Mares, Tosi, Poli, Andreotti, & Romagnoli, 2004). Based on the importance of medicinal plants, it was discovered that in Pakistan it is estimated that an 80% of its population depend on plant to cure themselves, a 40% in China. In technologically advanced countries as the United States, it is estimated that 60% of its population use medicinal plants habitually to fight certain ailments.

Modern medicine, through clinical tests, has been able to validate those plants that the tradition had used with the method of test and error. Many turned out to have been worth: potentially dangerous (McKay & Blumberg, 2006). The medicinal plants find application in pharmaceutical, cosmetic, agriculture and food industry. The use of the medicinal plants for curing disease has been documented in history of all civilizations. Man in the pre-historic era was probably not aware about the health hazards associated with irrational therapy. With the onset of research in medicine, it was concluded that plants contain active principles, which are responsible, for curative action of the herbs (Jeff-Agboola & Onifade, 2016). Medicinal and Aromatic plants form a numerically large group of economically important plants which provide basic raw materials for medicines, perfumes, flavours and cosmetics. The plants and their products not only serve as valuable source of income for small holders and entrepreneurs but also help the country to earn valuable foreign exchange by way of export (Sultabhaha, Suttajit, & Niyomca, 1992).

Aflatoxins are primarily produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*, which contaminate a wide variety of food and feed commodities including maize, oilseeds, spices, ground-nuts, tree nuts, milk and dried fruits. The presence of aflatoxins in food chain is associated with decrease in quality and quantity of food and feed materials. In addition, consumption of aflatoxin-contaminated products can pose a risk of development of various diseases in human and animals. *Aspergillus* can attack crops at different times, in the field, during harvest, transport and storage. Today, there are strict regulations on chemical pesticide use, and there is political pressure to remove the most hazardous chemicals from the market. Therefore, the study was designed to;

- Analyse and determine the effectiveness of four medicinal plants (*Cymbopogon citratus*, *Moringa oleifera*, *Clerodendrum volubile* and *Ocimum gratissimum*) against toxigenic A. flavus.
- Evaluate the minimum inhibitory concentration of the plants extracts against the toxigenic *A. flavus.*
- Investigate the phytochemical properties of the plant extracts.

2. Materials and method

2.1. Sample collection

Fresh leaves of *C. citratus, M. oleifera, C. volubile, O. gratissimum* (Figures 1–4) were collected from Abusoro area at Idepe Town, Okitipupa Local government, Ondo State, Nigeria. The leaves collected



Figure 1. The tree of *M. oleifera*.

Figure 2. Cymbopogon citratus.



Figure 3. Ocimum gratissimum.

were dried at 60°C using hot air oven for 96 h and thereafter grounded into powdered using warning blender. The toxigenic *A. flavus* used was isolated from contaminated poultry feed (Onifade, Jeff-Agboola, & Adesida, 2010).

2.2. Preparation of media

About 5.8 g of potato dextrose agar (PDA) was added to 150 ml of distilled water and then sterilized in autoclave at 121°C for 15 min. The sterilized media was poured into petri dishes at 45°C and allow to solidify. Holes were bored with 5 mm diameter cork bearer in the solidified agar.

Figure 4. Clerodendrum volubile.



2.3. Preparation of ethanolic, hot and cold water extract from the leaf samples

2.3.1. Cold water extraction method

Cold water extraction method was carried out to stimulate the traditional decoction method of preparing herbal preparation. Thirty grams of the ground powder was soaked in 300 ml sterile distilled cold water for 1 h and placed in a shaker at 100 rpm for 24 h at 25°C. The resulting elute was membranes-filtered (Onifade et al., 2010).

2.3.2. Hot water extraction method

Thirty grams of the ground powder was soaked in 300 ml water and boiled for 30 min and was allowed to stand for 24 h at 25°C. The resulting elute was membranes-filtered (Onifade et al., 2010).

2.3.3. Ethanol extraction method

Thirty grams of the ground leave powder was soaked for 72 h in a beaker containing 300 ml of ethanol and placed in a shaker at 100 rpm for 72 h at 25°C. The resulting extracts was membrane filtered on a Whitman paper number 1 then the filtrate concentrated at 50°C using a vacuum rotary evaporator. In order to evaporate the ethanol used as the extraction solvent, the concentration was air dried at 25°C until a constant weight was attained. A stock solution of the ethanol-free past was prepared by dissolving 0.4 g in 20 ml of deionised sterile distilled water (Jeff-Agboola, Onifade, Akinyele, & Osho, 2012).

2.3.4. Pre-screening of the extracts for antifungal activity against toxigenic A. flavus

Preliminary screening of the extracts for antifungal effect was done using toxigenic *A. flavus* as the test organism. Sterile PDA was poured in a petri-dish and allowed to solidify. Almost 100 µl of 10⁶ (Cfu/ml) of toxigenic *A. flavus* was dispensed at the centre of the sterile agar. It was spread with sterile glass spreader.

Therefore, 5 mm in diameter of cork-borer was used to bore hole in the agar. Then 200 μ l of the plant extract were placed in the hole. Extract-free sterile distilled water, was inoculated in another set of plates and used as negative controls. The plates were allowed to stand for at least 1 h at 25°C for the extracts to diffuse at the point of inoculation before incubation at 37°C for 8 h. Observation of a clear zone on the fungal plate at the point of inoculation of the extract was interpreted as

evidence of inhibition of mould growth. The diameter of these zones was measured and recorded (Daniyan & Muhammad, 2008).

2.3.5. Determination of the efficacy of the plant extracts

Different concentrations of the plant extract were as eptically dispensed into the well bored on the agar and were incubated at $\pm 28^{\circ}$ C for 72 h and beyond.

2.3.6. Determination of minimum inhibitory concentrations

The initial concentration of the plant extract (100 mg/ml from 30 g into 300 ml) was diluted using double fold serial dilution by transferring 5 ml of the sterile plant extract (stock solution) into 5 ml of sterile Nutrient broth to obtain 50 mg/ml concentration. The above process was repeated several times to obtain other dilutions: 25, 12.5, 6.25, and 3.125 mg/ml (Rasooli & Abyaneh, 2004). Having obtained the different concentrations of the extracts, each concentration was inoculated with 0.1 ml of the standardised spore suspension and incubation was done at 30°C for 72 h. The growth of the inoculum in the broth is indicated by turbidity or cloudiness of the broth and the lowest concentration of the extract which inhibited the growth of the test organism were taken as the MIC (Jeff-Agboola & Onifade, 2016).

2.3.7. Phytochemical screening

Phytochemical examinations were carried out for all the extracts according to standard methods (Harborne, 1973; Odebiyi & Sofowora, 1978; Onwukeame, Ikuegbvweha, & Asonye, 2007; Sofowora, 1982).

2.3.8. Detection of tannins

To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

2.3.9. Detection of flavonoids

Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

2.3.10. Detection of glycosides

Extracts were hydrolysed with dilute HCl, and then subjected to test for glycosides. Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 min. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

2.3.11. Detection of saponins

Extracts were diluted with distilled water to 20 ml and this was shaken in a graduated cylinder for 15 min. Formation of 1 cm layer of foam indicates the presence of saponins.

2.3.12. Data analysis

The experiment was conducted using a completely randomized design. Means of three replicates were computed using computer software Microsoft Excel.

3. Results

The results of the antifungal assay of the plant extracts indicated that these plant exhibited antifungal activity against toxigenic *A. flavus* at different time interval, and the potential sensitivity of the extract was obtained against toxigenic *A. flavus* and the zone of inhibition were recorded.

The result obtained in antifungal testing of the plant extracts shows that the effectiveness of each plant extract varies with time and also some plant extract were not effective against tested organism based on the method of extraction.

The result obtained from agar diffusion method for ethanolic extraction method (Table 1) shows that *C. citratus* (0.00 mm) has no effect on toxigenic *A. flavus* while *M. oleifera* (12.00 mm), *O. gratissimum* (14.00 mm), and *C. volubile* (15.00) had inhibitory effect on toxigenic *A. flavus*. However, the mode of action of *M. oleifera* (12.00 mm) and *O. gratissimum* (14.00 mm) are fungistatic while the mode of action of *C. volubile* (15.00 mm) on toxigenic *A. flavus* was fungicidal.

The result obtained from agar diffusion method from cold-water extracts (Table 2) shows that *C. citratus* (0.00 mm) and *O. gratissimum* (0.00 mm) has no inhibitory effect on the toxigenic *A. flavus while M. oleifera* (16.00 mm) and *C. volubile* (13.00 mm) has effect on toxigenic *A. flavus.* However, it was noted that the effectiveness of *M. oleifera* (16.00 mm) was fungicidal while the effect of *C. volubile* (13.00 mm) was fungistatic.

The result of the hot-water extracts shows that *C. citratus* (10.00 mm), *M. oleifera* (11.00) and *O. gratissimum* (11.00 mm) (Table 3) had inhibitory effect on toxigenic *A. flavus* while and *C. volubile* (0.00 mm) has no effect on the toxigenic *A. flavus*. Effectiveness of plant extracts from hot water extractive method was observed to be fungistatic.

Table 1. The result of the agar diffusion method (mm) of ethanolic plant extracts againstA. flavus at different time interval

Plant extracts	Hours			Mode of action	
	24 h	48 h	72 h		
Cymbopogon citratus	0.00	0.00	0.00	Not effective	
Moringa oleifera	11.70	11.80	12.00	Fungistatic	
Ocimum gratissimum	15.00	15.00	14.70	Fungicidal	
Clerodendrum volubile	15.00	15.00	15.00	Fungicidal	

Table 2. The result of the agar diffusion method (mm) of cold water plant extracts against *A. flavus* at different time interval

Plant extracts	Hours			Mode of action	
	24 h	48 h	72 h		
Cymbopogon citratus	0.00	0.00	0.00	Not effective	
Moringa oleifera	15.80	16.00	16.00	Fungicidal	
Ocimum gratissimum	0.00	0.00	0.00	Not effective	
Clerodendrum volubile	12.80	13.00	13.00	Fungistatic	

Table 3. The result of the agar diffusion method (mm) of hot-water plant extracts against *A. flavus* at different time interval

Plant extracts	Hours			Mode of action	
	24 h	48 h	72 h		
Cymbopogon citratus	9.80	10.00	10.00	Fungistatic	
Moringa oleifera	10.90	10.90	11.00	Fungistatic	
Ocimum gratissimum	11.00	11.00	11.00	Fungistatic	
Clerodendrum volubile	0.00	0.00	0.00	Not effective	

Table 4. Minimum inhibitory concentration					
Plant extracts	Ethanolic extract (mg/ml)	Cold-water extract (mg/ml)	Hot-water extract (mg/ml)		
Cymbopogon citratus	_	-	6.25		
Moringa oleifera	12.5	50	12.5		
Ocimum gratissimum	25	-	25		
Clerodendrum volubile	50	25	-		

The result obtained on minimum inhibitory concentration (Table 4) shows that 50 mg/ml concentration of ethanolic extract of *C. volubile* had fungicidal effect on toxigenic *A. flavus*; 25 mg/ml concentration of ethanolic extract of *O. gratissimum* had fungicidal effect on toxigenic *A. flavus*, while 12.5 mg/ml concentration of ethanolic extract of *M. oleifera* had fungistatic effect on toxigenic *A. flavus*, while 12.5 mg/ml concentration of ethanolic extract of *M. oleifera* had fungistatic effect on toxigenic *A. flavus*, while 12.5 mg/ml concentration of ethanolic extract of *M. oleifera* had fungistatic effect on toxigenic *A. flavus*. These results indicated that among tested ethanolic plant extracts, there was a significance difference. In other words, *M. oleifera* shows the highest inhibitory effect on toxigenic *A. flavus*, while *C. citratus* shows the least. Experiments related to the 1,000 µg/ml concentration effect of leaf essential oil against the tested organism shows that *M. oleifera*, *O. gratissimum*, and *C. volubile* showed inhibitory effects while no inhibitory effect from *C. citratus* was observed.

From cold-water method of extraction, 50 mg/ml concentration of cold water extract of *M. oleifera* had fungicidal effect on toxigenic *A. flavus*, while 25 mg/ml concentration of cold water extract of *C. volubile* had fungistatic effect on toxigenic *A. flavus*. These results indicated that among tested cold water plant extracts, there was a significance difference. *C. volubile* shows the highest inhibitory sensitivity to toxigenic *A. flavus*, while *M. oleifera* shows the least. Experiments related to the 1,000 µg/ml concentration effect of leaf essential oil against the tested organism shows that *C. volubile*, and *M. oleifera* showed inhibitory effects while no inhibitory effect from *C. citratus* and *O. gratissimum* were observed.

From hot-water method of extraction, 25 mg/ml concentration of hot-water extract of *O. gratis-simum* had fungicidal effect on toxigenic *A. flavus*, 12.5 mg/ml concentration of hot water extract of *M. oleifera* while 6.25 mg/ml concentration of hot water extract of *C. citratus*.

The results obtained from phytochemical analysis for saponins (Table 5) shows that saponins was not present in *C. citratus* while the result shows the presence of saponins in *M. oleifera*, *O. gratissimum*, and *Clerodendrum* spp.

The result obtained from phytochemical analysis for tannins (Table 5) shows that tannins was present in plant extracts of *C. citratus*, *M. oleifera*, *O. gratissimum*, and *C. volubile* respectively.

The result obtained from phytochemical analysis for volatile oils (Table 5) shows the absence of volatile oils in all the plant extracts *C. citratus*, *M. oleifera*, *O. gratissimum*, and *C. volubile* irrespective of their method of extractions.

The result obtained from phytochemical analysis for glycosides shows the absence of glycosides in all the plant extracts *C. citratus*, *M. oleifera*, *O. gratissimum*, and *C. volubile* irrespective of their method of extractions.

The result of the determination of minimum inhibitory concentration shows that ethanolic extract of 50 mg/ml of *C. volubile*, 25 mg/ml of *O. gratissimum* and 12.5 mg/ml of *M. oleifera* had a fungicidal effect on *A. flavus*, cold water extract of 50 mg/ml of *M. oleifera*, and 25 mg/ml of *C. volubile* had effect on toxigenic *A. flavus* while Hot water extract of 25 mg/ml of *O. gratissimum*, 12.5 mg/ml of *M. oleifera* and 6.25 mg/ml of *C. citratus* had effect on toxigenic *A. flavus*. These results indicated that among tested hot water extracted plant extract, there was a significance difference. *C. citratus*

Table 5. Phytochemical screening of the medicinal plant extracts					
	Ethanolic extract	Cold-water extract	Hot-water extract		
A. Phytochemical screening o	f plant extracts for saponins				
Cymbopogon citratus	-	-	-		
Moringa oleifera	+	+	+		
Ocimum gratissimum	+	+	+		
Clerodendrum volubile	+	+	+		
B. Phytochemical screening o	f plant extracts for tannins				
Cymbopogon citratus	+	+	+		
Moringa oleifera	+	+	+		
Ocimum gratissimum	+	+	+		
Clerodendrum volubile	+	+	+		
C. Phytochemical analysis of	plant extracts for volatile oils				
Cymbopogon citratus	-	-	-		
Moringa oleifera	-	_	-		
Ocimum gratissimum	-	-	-		
Clerodendrum volubile	-	-	-		
D. Phytochemical analysis of	plant extracts for glycosides				
Cymbopogon citratus	-	-	_		
Moringa oleifera	-	-	-		
Ocimum gratissimum	_	-	_		
Clerodendrum volubile	_	_	_		

shows the highest inhibitory sensitivity to toxigenic *A. flavus*, while *M. oleifera* and *O. gratissimum* shows the least. Experiments related to the 1,000 µg/ml concentration effect of leaf essential oil against the tested organism shows that *C. citratus*, *M. oleifera* and *O. gratissimum* showed inhibitory effects while no inhibitory effect from *C. volubile* were observed.

The results of phytochemical screening of the plant extracts for the detection of volatile oil, glycoside, tannins and saponins shows that saponin was present in the ethanolic, cold and hot-water extract of *M. oleifera*, *O. gratissimum* and *C. volubile* with the exception of *C. citratus* (Table 5). Tannins was also present in the ethanolic, cold and hot-water extract of *M. oleifera*, *O. gratissimum*, *C. volubile* and *C. citratus* (Table 5). Volatile oil and glycoside were not detected in all the plant extracts.

4. Discussion

In recent years much research has been conducted in the field of antifungal effects of different plants. In the present investigation, the antifungal activity of the plant extracts was assayed against toxigenic *A. flavus* at different time interval but with the same concentration of extracts to understand the most effective activity.

Several workers have made similar observations by using essential oils or complex mixture from higher plants (Gonzalez-Lamothe et al., 2009). Some medicinal plants have higher antifungal properties and higher diffusion power (Gonzalez-Lamothe et al., 2009).

Investigators in the past had also clearly shown that ethanolic extracts were more effective than water extract (Bakht, Tayyab, Ali, Islam, & Shafi, 2011). They have attributed this observation to the high volatility of ethanol which tends to extract more active compound from the sample than water. Hence, these studies followed similar trends.

Several investigators studied the antifungal activity of essential oil of *C. citratus, M. oleifera, O. gratissimum* and *C. volubile* plant against toxigenic *A. flavus.* A study by some researchers (Onifade et al., 2010; Jeff-Agboola et al., 2012) has concluded that essential oil prepared from *C. citratus* maybe a safe alternative environment inhibition of antifungal agents for various uses. It seems that generally antifungal properties of ethanolic extracts, cold-water extracts and hot-water extracts can be attributed to the presence of secondary metabolites especially flavonoids in first degree, in the second degree terpenes and in the third degree saponins.

The efficiency of the antifungal effect of the plant extract against toxigenic *A. flavus* using different extraction methods.

The increase in the antifungal activity of the extracts was enhanced by increase in the concentration of the extracts. This finding agrees with the report of Mares et al. (2004) that higher concentration of antimicrobial substance showed appreciation in growth inhibition.

The fact that the results of this study showed that plants extracts that exhibited antifungal properties justifies their traditional use as medicinal plants. This may be due to the presence of active principles in the plant materials. Plants generally produce many secondary metabolites which constitute an important source of microbicides, pesticides and many pharmaceutical drugs (de Billerbeck, Roques, Bessière, Fonvieille, & Dargent, 2001). Plant products still remain the principal source of pharmaceutical agents used in orthodox medicine (de Billerbeck et al., 2001).

The minimum inhibitory concentration values of the plant extracts against the test organism showed that fungi vary widely in the degree of their susceptibility to antifungal agents.

Several reports stated that the extracts of medicinal plants play an important role in controlling many phytopathogenic fungi (Abd-El-Khair & Haggag, 2007; Choi et al., 2004; Lin, Zon, Lin, & Tan, 2001; Okemo, Bias, & Vivanco, 2003; Perez-Sanchez, Infante, Galvez, & Ubera, 2007). The inhibitory effect of some medicinal plants might be due to the presence of steroids, terpenoids, alkaloids, citral, geraniol, flavonoids, eugenol, cytronolal, geranyl acetate, beta cariofiln, tannins, phenolic compounds, saponins and farnsul.

5. Conclusion

These findings suggest a new pathway in elucidating a potent antifungal agent from plants like *C. citratus, M. oleifera, O. gratissimum,* and *C. volubile.* The Present study indicates that the plant contains antifungal compound that can be further developed as phytomedicine for the therapy of infection. Such screening of various natural organic compounds and identification of active agents is the need of the hour because successful prediction of lead molecule at the onset of drug discovery will pay off later in drug development.

In conclusion, the action of extracts upon the antifungal models justified its usefulness in herbal formulation. In conclusion, the results obtained from this study shows that the plants extracts used in this study exhibit antifungal activities against toxigenic *A. flavus*. Extracts of the plant used in this study could be useful in the treatment of fungal infections caused by *A. flavus* (Jeff-Agboola et al., 2012).

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